REMARKS

Upon entry of this amendment, Claims 1, 3-5, and 14-41 constitute the pending claims in the present application. Among them, Claims 3, 28, and 29 are directed to non-elected species, and are withdrawn from further consideration. Claims 6-13 are canceled without prejudice. Applicants reserve the right to prosecute claims of identical or similar scope in future continuation and/or divisional applications.

Applicants note that the IDS received on October 2, 2006 has been considered by the Examiner.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the Office Action.

Application Status

Applicants note that, in the Office Action Summary, the instant Office Action is a non-Final Office Action. This is consistent with the fact that the newly raised enablement rejection with respect to Claims 35, 36, and 38-40 is "a new ground of rejection that is neither necessitated by applicant's amendment of the claims nor based on information submitted in an information disclosure statement filed during the period set forth in 37 C.F.R. § 1.97(c) with the fee set forth in 37 C.F.R. § 1.17(p)." MPEP 706.07(a).

However, page 13 of the Office Action indicates that "**THIS ACTION IS MADE FINAL.**"

Applicants' attorney also reviewed public PAIR, and found that under the Application Data tab, the status of the application is "Final Rejection Mailed," while under the Image File Wrapper Tab, the most recent action is "non-Final Rejection" mailed.

In an attempt to resolve the issue, Applicants' attorney Yu Lu contacted Examiner Meah via telephone on May 16, 2007. During the telephone call, the Examiner was unable to confirm that the instant Office Action is a Final action. After hearing Applicants' argument that the current enablement rejection is neither necessitated by Applicants' amendment of the claims nor

based on IDS filed during the period set forth in 37 C.F.R. § 1.97(c) with the fee set forth in 37 C.F.R. § 1.17(p), the Examiner acknowledged that there might have been a mistake as to the status of the application. The Examiner suggested Applicants to argue for the withdrawal of the finality of the Office Action.

Applicants submit that the Office Action Summary form PTOL-326 (rather than the form paragraph on page 13 of the Office Action) should control the status of the application. *See* MPEP 707. Particularly in this case, the rejection could not have been made Final per MPEP 706.07(a). **Thus Applicants treat this application as being non-Finally rejected, and respond under 37 C.F.R. § 1.111.**

In addition, this response also constitutes the required "complete written statement of the reasons presented at the interview as warranting favorable action" under 37 C.F.R. § 1.133(b).

In the alternative, if the Examiner insists that the current Office Action is a Final action, Applicants submit that the Final rejection is improper with respect to the new ground of 35 U.S.C. 112, first paragraph (enablement) rejection. Such new ground of rejection is neither necessitated by Applicants' amendment of the claims nor based on information submitted in an IDS filed during the period set forth in 37 C.F.R. § 1.97(c) with the fee set forth in 37 C.F.R. § 1.17(p). *See* MPEP 706.07(a). Applicants respectfully request the Examiner to reconsider and withdraw the finality of the Office Action, so as to allow Applicants the opportunity to respond to the new ground of rejection.

Claim Rejections under 35 U.S.C. § 112, second paragraph

The Office Action rejects Claims 8-16, 31, 32, and 36 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. Specifically, the Office Action alleges that the term "potency" makes claims confusing, and requires Applicants to change it to the "commonly known term in (the) art" (emphasis original) such as "enzyme activity."

The Examiner seems to argue that only "commonly known terms in the art" should be used in the claims, and "potency" is not such a commonly known term. Applicants respectfully disagree.

"A fundamental principle contained in 35 U.S.C. 112, second paragraph is that applicants are their own lexicographers. They can define in the claims what they regard as their invention essentially in whatever terms they choose ..." MPEP 2173.01.

"The examiner's focus during examination of claims for compliance with the requirement for definiteness of 35 U.S.C. 112, second paragraph, is whether the claim meets the threshold requirements of clarity and precision, not whether more suitable language or modes of expression are available. When the examiner is satisfied that patentable subject matter is disclosed, and it is apparent to the examiner that the claims are directed to such patentable subject matter, he or she should allow claims which define the patentable subject matter with a reasonable degree of particularity and distinctness. Some latitude in the manner of expression and the aptness of terms should be permitted even though the claim language is not as precise as the examiner might desire. Examiners are encouraged to suggest claim language to applicants to improve the clarity or precision of the language used, but should not reject claims or insist on their own preferences if other modes of expression selected by applicants satisfy the statutory requirement" (emphasis original). MPEP 2173.02.

Therefore, all that is required by the statute is a "reasonable degree of particularity and distinctness."

"If the language of the claim is such that a person of ordinary skill in the art could not interpret the metes and bounds of the claim so as to understand how to avoid infringement, a rejection of the claim under 35 U.S.C. 112, second paragraph, would be appropriate. *See Morton Int'l, Inc. v. Cardinal Chem. Co.*, 5 F.3d 1464, 1470, 28 USPQ2d 1190, 1195 (Fed. Cir. 1993). However, if the language used by applicant satisfies the statutory requirements of 35 U.S.C. § 112, second paragraph, but the examiner merely wants the applicant to improve the clarity or precision of the language used, the claim must not be rejected under 35 U.S.C. § 112, second paragraph, rather, the examiner should suggest improved language to the applicant. ... If applicant does not accept the examiner's suggestion, the examiner should not pursue the issue." *Id.*

While Applicants appreciate the Examiner's suggestion intended to improve the claim

language, MPEP clearly states that the Examiner "should not pursue the issue" if Applicants choose to use their own terminology that satisfies the statutory requirement. Accordingly, reconsideration and withdrawal of the rejections are respectfully requested.

Claim Rejections under 35 U.S.C. § 112, first paragraph

Claims 6-13, 31, 35, 36, 38-40 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to meet the enablement requirement.

Specifically, Claims 6-13 and 31 remain rejected because these claims recite kinetic properties that allegedly render the scope of the claims not commensurate with the enablement provided by the disclosure. The Office Action argues that the claims encompass an extremely large number of adzymes, and the specific kinetic parameters depend on multiple factors, thus "achievement of desired kinetic values for the broad class of adzymes ... acting on [sic] board class of substrates is highly unlikely."

Applicants respectfully disagree with the Examiner's reasoning. After all, for any address domain conjugated to the catalytic domain, a skilled artisan could easily use random mutagenesis coupled with screening to identify adzymes with desired / claimed kinetic parameter, using no more than routine experimentation. However, solely for the purpose of advancing prosecution, Applicants have canceled Claims 6-13 to obviate this rejection.

Furthermore, Applicants submit that Claim 31 is different from Claims 6-13, in that it does not recite any kinetic parameters of the claimed adzymes. The numbers recited in the claims relate to assay conditions (*e.g.*, concentrations of adzyme substrates and the potentially interfering serum proteins). Thus the rationale proffered (*e.g.*, requiring undue experimentation by skilled artisan to achieve the recited kinetic parameters of the claim adzymes) does not seem to support the rejection of Claim 31.

Claims 35 and 36 are rejected under 35 U.S.C. § 112, 1st paragraph, for allegedly failing to meet the enablement requirement. The Office Action asserts that Claims 35 and 36 "broadly recite the use of any substrate polypeptide, which is cleaved by adzyme to produce any product that inhibits the substrate binding or adzyme cleavage," while the specification "fails to describe

in any fashion the physical and/or chemical properties of the claimed class of substrates and their by-products ..." This is a new ground of rejection not raised in the previous Office Action.

Applicants again respectfully disagree with the Examiner's reasoning. It is well-known in the art that many molecules, after undergoing proteolysis, may become a so-called "dominant negative" version ("DN") of the wild-type molecule ("WT"), in the sense that DN competes with WT to bind the cognate binding partner and interferes with WT function. One such example relates to soluble receptor for cytokines. Such soluble receptors may be generated by proteolytic cleavage of the membrane-bound WT receptors (*see* page 2569, left column, first full paragraph in Fernandez-Botran, *The FASEB J.* **5**: 2567-2574, 1991, **Exhibit A**). Once cleaved, the soluble receptor (DN) competes with the membrane-bound WT receptor for binding to the circulating cognate cytokine, thus preventing the cytokine from binding to the WT receptor (and antagonizing WT receptor function).

In a similar fashion, a cleaved cytokine may also become a dominant negative version of the wild-type cytokine, in that the cleaved cytokine competes with the wild-type cytokine for binding to the cognate cytokine receptor (but fails to trigger receptor dimerization, *etc.*), thus antagonizing the function of the wild-type cytokine.

The same argument also applies to many other molecules involved in ligand-receptor interaction, such as extracellular signaling molecules, receptors, adhesion molecules, *etc.*

Thus, a skilled artisan could readily use no more than routine experimentation to identify an adzyme that cleaves its substrate to produce a cleavage product, which resembles the substrate (*e.g.*, a signaling peptide or a receptor) but lacks the biological activity of the substrate (*e.g.*, lacks the ability to dimerize receptor, or transmit downstream signaling, respectively).

"A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984)." MPEP 2164.01.

Therefore, based on the teaching of the instant specification, coupled to what is known in

the art, a person of ordinary skill in the art would be able to generate adzymes that produce antagonistic cleavage products.

Claims 38-40 are rejected under 35 U.S.C. § 112, 1st paragraph, for allegedly failing to meet the enablement requirement. The Office Action asserts that these claims "recite an adzyme composition wherein autocatalytic proteolysis is inhibited by any means or especially by inclusion of a reversible inhibitor but it is not clear that there are available reversible inhibitors for any protease nor are other means of formulating an adzyme composition to present auto proteolysis is taught..." This is a new ground of rejection not raised in the previous Office Action.

Applicants respectfully disagree, because there are numerous art-recognized reversible protease inhibitors, many (if not all) are commercially available. For example, Sigma-Aldrich sells numerous broad-spectrum protease inhibitors, such as Serine protease inhibitors, Cysteine protease inhibitors, Aspartic protease inhibitors, and Metalloproteinase inhibitors, including Leupeptin, PMSF, AEBSF, Aprotinin, Chymostatin, Antithrombin III, 3,4-Dichloroisocoumarin, TLCK, TPCK, DIFP, Antipain, α2-Macroglobulin, N-Ethylmaleimide, E-64, Pepstatin A, EDTA, 1,10-Phenanthroline, Phosphoramidon, Bestatin, *etc.* *See* http://www.sigmaaldrich.com/Area_of_Interest/Biochemicals/Enzyme_Explorer /Key_Resources/Protease_Inhibitors/Broad_Spectrum_Inhib_.html.

"A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984)." MPEP 2164.01.

Furthermore, contrary to the Office Action's assertion, the instant specification (from page 114 to page 117) describes various means that may be used to prevent autocatalysis.

Therefore, based on the teaching of the instant specification, coupled to what is known in the art, a person of ordinary skill in the art would be able to formulate the claimed pharmaceutical preparation to prevent autocatalysis, for example, by using a myriad of available

reversible protease inhibitors.

In view of the foregoing, all pending claims satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim Rejections under 35 U.S.C. § 102

The Office Action rejects Claims 1, 4-27, 30, 34, 37, 38, and 41 under 35 U.S.C. § 102 as allegedly being anticipated by Holvoet *et al.* (JBC 266: 19717-24, 1991, "Holvoet") or Davis *et al.* (WO 00/64485, "Davis") or Chen *et al.* (US 2003/0068792, or "Chen").

In response to Applicants' previous argument that Holvoet, Davis, or Chen does not teach a fusion protein resistant to autoproteolytic cleavage, the Office Action argues that the cited references teach polypeptides that "must inherently be resistant to self-cleavage," because "there is no available evidence to suggest that they are labile to autoproteolysis and furthermore as their fusion proteins are stable enough to show protease activity to cleave substrate polypeptide." Applicants respectfully disagree, not only for the inherency theory of anticipation advanced by the Examiner, but also for the reasons below.

Specifically, Holvoet describes a fusion protein comprising a urokinase-type plasminogen activator (uPA) and a fibrin-specific antibody. The fusion protein binds the fibrin clot via its fibrin-specific antibody, but the uPA catalytic domain does not cleave the fibrin clot. Instead, it cleaves the soluble zymogen plasminogen in the circulating blood to yield an active Ser protease plasmin, which in turn dissolves the fibrin clot. In other words, Holvoet describes a fusion protein comprising an targeting domain (*i.e.*, the fibrin-specific antibody) that does not bind the substrate (*i.e.*, the plasminogen) which the catalytic domain (uPA) cleaves. In contrast, the claimed invention requires the targeting domain to bind the same substrate which the catalytic domain cleaves.

Therefore, Holvoet cannot anticipate the claimed invention.

Chen relates to a so-called "targeted enzyme" that "comprises a substrate recognition site and has been modified from a pre-targeted enzyme to comprise one or more targeting sites, each

targeting site comprising one or more variant sequences, and to bind to a target with higher affinity than the corresponding pre-targeted enzyme binds the target under like conditions. ... Targeted enzymes of the invention do not include enzymes with a targeting site that consists of a polypeptide or other target-binding molecule that is attached to the N- or C-terminus of the pre-targeted enzyme (e.g., as in a histidine tagged protein or a fusion protein), a targeted enzyme whose only target is a monoclonal antibody, or a targeted enzyme made by increasing or optimizing the binding of a pre-targeted enzyme to a substrate of a reaction catalyzed by the pre-targeted enzyme" (emphasis added).

In other words, the "targeted enzyme" of Chen merely modifies so-called "variant sequences" on the pre-targeted enzyme, such that after the modification, the enzyme acquires the ability to bind a target that the enzyme previously cannot bind. In doing so, Chen explicitly states that the "targeted enzyme" does not include any fusion protein created by fusing a targeting domain to (the catalytic domain of) the enzyme (see emphasis above), as is recited in the claimed invention.

Thus Chen cannot anticipate the claimed invention, because the "targeted enzyme" in Chen is not a fusion protein or immunoglobulin fusion complex with discrete and heterologous protease domain and targeting domain, as recited in the claimed invention.

Likewise, Davis teaches away from the claimed fusion protein by suggesting that the targeting moiety should be coupled to the catalytic domain through chemical cross-linking to a Cysteine sulfur in polypeptide comprising the catalytic domain. *See* page 6, 1st full paragraph, and the paragraph bridging pages 21 and 22.

Thus Davis cannot anticipate the claimed invention, because the chemically cross-linked modified enzyme in Davis is not a fusion protein as recited in Claim 1 or an immunoglobulin fusion complex as recited in Claim 41.

Reconsideration and withdrawal of the rejections are respectfully requested.

Claim Rejections under 35 U.S.C. § 103

The Office Action rejects Claims 1, 4, 14-17, 18-27, 30-38, and 41 under 35 U.S.C.

§ 103(a) as allegedly being obvious in view of Holvoet or Davis or Chen or Guo *et al.* (*Biotec. and Biong* **70**: 456-463, 2000, or "Guo") in view of Sallberg *et al.* (U.S. Pat. No. 6,960,569) or Whitcomb.

The Office Action argues that "Whitcomb *et al.* ([sic] US PAT4510251) teach mesotrypsin – a trypsin-like protease (page 10, 1st paragraph) that is fairly stable to proteolytic cleavage and also teach that mesotrypsin rapidly degrades and inactivate zymogens and other polypeptides. Sallberg *et al.* (US 6960569) teach fusion protein of mutated NS3/4A protease domain of HCV conjugated to antibody or other protein wherein fusion protein is resistant to proteolytic cleavage (mutation of breaking point residues of protease causes resistance to the proteolytic cleavage)."

Thus, the Examiner concludes that "... it would have been obvious to one of ordinary skill in the art to use mesotrypsin – a trypsin-like protease that is fairly stable [sic] to proteolytic cleavage as taught by Whitcomb *et al.* or mutation of protease as taught by Sallberg and conjugate said proteases by a linker as taught by Guo *et al.* to target domain as taught by Holvoet ... or Davis ... or Chen and use the resulting adzyme to inactivate substrate polypeptides by catalyzing the proteolytic cleavage of [sic] the said substrate polypeptide."

In response to the recent U.S. Supreme Court decision *KSR Int'l Co. v. Teleflex, Inc.*, No. 04-1350 (U.S. Apr. 30, 2007), Ms. Margaret A. Focarino, the Deputy Commissioner for Patent Operations, sent a memorandum dated May 3, 2007 to the USPTO Technology Center Directors. In that memo, Ms. Focarino states that the Court reaffirmed the four factual inquiries under *Graham* in the determination of obviousness under 35 U.S.C. § 103(a), and that the Court did not totally reject the use of "teaching, suggestion, or motivation" as a factor in the obviousness analysis. At the end of the memo, she emphasizes that "... **in formulating a rejection under 35 U.S.C. § 103(a) based upon a combination of prior art elements, it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed**" (emphasis original).

This is consistent with *KSR*, where the Court states that:

> "Often, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. **To facilitate review, this analysis should be made explicit**. *See In re Kahn*, 441 F. 3d 977, 988 (CA Fed. 2006) ('**[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness**')" (emphasis added).

Applicants submit that the Office Action has not satisfied the requirement of establishing a *prima facie* case of obviousness by explicitly stating articulated reasoning for combining the cited references, as is required by case law. Other than the conclusory statements, the only reason the Office Action proffered in support of the rejection is that mesotrypsin is "fairly stable to proteolytic cleavage" as allegedly taught by Whitcomb. Applicants, however, are unable to find where in Whitcomb is the alleged teaching that mesotrypsin is "fairly stable to proteolytic cleavage." In addition, the Office Action does not offer *any* reason as to why Sallberg may be combined with any of the other references.

Specifically, Whitcomb only has 14 columns of text, Applicants assume "page 10 1st paragraph" recited in the Office Action means "column 10, 1st paragraph (lines 1-30)." In that paragraph, Whitcomb first describes mesotrypsin as one of the two "trypsin-like proteases that are activated by trypsin, and in turn rapidly degrade trypsin and other zymogens." Whitcomb then describes mesotrypsin as being "completely resistant to PSTI neutralization," and that mesotrypsin can hydrolyze zymogen even in the presence of PSTI.

It is evident that the first sentence merely describes the activation of mesotrypsin by trypsin, and the biochemical function of mesotrypsin (*i.e.*, degrade zymogen, *etc.*). It is completely silent about the stability of mesotrypsin itself, *e.g.*, whether mesotrypsin is susceptible to autocatalysis or proteolysis by other enzymes. Likewise, the second sentence also has nothing to do with the stability of mesotrypsin. Note that PSTI is not a protease, and thus it does not inhibit the activity of trypsin by proteolytically cleave trypsin or mesotrypsin, as the Office Action apparently suggests. Instead, PSTI merely binds to proteases like trypsin and inhibits the protease activity of trypsin (*see* col. 8 and 9 of Whitcomb). It is unclear, based on

Whitcomb, whether PSTI binds to mesotrypsin but fails to inhibit its activity, or fails to bind mesotrypsin at all.

Therefore, the Office Action has failed to provide a valid reason why a person of ordinary skill in the art would have combined Whitcomb with the other cited references in an attempt to reach the claimed invention.

Regarding Sallberg, Applicants were unable to find any disclosure in Sallberg that relate to conjugating the NS3/4A fusion protein to antibody or other protein, as the Office Action alleges. Sallberg relates to the identification of a new NS3/4A fusion of the HCV virus, its truncation mutants, or its mutations that lack a proteolytic cleavage site. *See* col. 3, lines 30-50. Sallberg contemplates the use of such HCV peptides as immunogens to generate antibodies against NS3 (*see* col. 3, last paragraph). Applicants, however, were unable to find any disclosure about any conjugates of such NS3/4A fusion to any antibodies or other proteins. In addition, although Sallberg disclose a proteolytic resistant mutant of the NS3/4A fusion protein, it is unclear whether the mutation occurs within the NS3 Ser protease domain or elsewhere in the NS3/4A fusion. Applicants note that in Example 3 (col. 14), Sallberg discloses a protease resistant mutant, wherein the protease resistant site is between the NS3 and NS4A fusion parts. This experiment shows that, while the protease resistant version of the NS3/4A mutant is still "comparable" to the wild-type NS3 protein in terms of immunogenicity, the NS3/4A fusion that is not protease resistant works better as an immunogen than the protease resistant version. "...the NS4A sequence and a functional proteolytic cleavage site between the NS3 and NS4A sequences provided for a more potent immune response" (emphasis added). *See* col. 3, Example 3 and Table 2.

Therefore, contrary to what the Office Action alleges, Sallberg does not disclose any NS3/4A fusion conjugated to any antibody or other possible targeting domains. In addition, it is unclear if Sallberg teaches any protease-resistant version of the NS3 protease, since the protease-resistant mutant of the NS3/4A fusion only appears to have a protease resistant site between the NS3 and NS4A parts (but not inside the NS3 protease domain). Sallberg also teaches away from using a protease-resistant version of the NS3/4A fusion (compared to the non-protease-resistant NS3/4A fusion), because the non-protease-resistant version is a better immunogen.

In view of the foregoing, the Office Action has failed to provide a single reason why it would have been obvious to substitute mesotrypsin disclosed in Whitcomb or the NS3 protease domain in Sallberg for any of the protease domains described in Holvoet, Davis, and Chen. Reconsideration and withdrawal of the obviousness rejection are respectfully requested.

Applicants further submit that, even for the sake of argument, assuming Whitcomb or Sallberg can be combined with Holvoet, Davis, or Chen, the combined teaching still fails to teach or suggest the claimed invention for missing one or more required claim elements. Applicants note that in *KSR*, regarding Teleflex's newly raised argument that the combined prior art fails to teach the claimed invention, the Supreme Court dismissed the argument since Teleflex failed "to raise the argument in a clear fashion (in the district court)." *KSR Int'l Co. v. Teleflex, Inc.*, Section III(A).

Specifically, as described above, Sallberg only appears to describe a protease-resistant mutant of the NS3/4A fusion protein, wherein the protease-resistant site is between the NS3 and NS4A parts, not within the NS3 protease domain. Applicants respectfully request the Examiner to point out where in Sallberg is the disclosure for a protease-resistant version of the NS3 protease domain. Thus even if Sallberg is combined with Holvoet, Davis, or Chen, the combined teaching fails to arrive at the recited autocleavage-resistant protease domain.

Regarding combination with Whitcomb, Holvoet describes a fusion protein comprising a urokinase-type plasminogen activator (uPA) and a fibrin-specific antibody. The fusion protein binds the fibrin clot via its fibrin-specific antibody, but the uPA catalytic domain does not cleave the fibrin clot. Instead, it cleaves the soluble zymogen plasminogen in the circulating blood to yield an active Ser protease plasmin, which in turn dissolves the fibrin clot. In other words, Holvoet describes a fusion protein comprising an targeting domain (*i.e.*, the fibrin-specific antibody) that does not bind the substrate (*i.e.*, the plasminogen) which the catalytic domain (uPA) cleaves. In contrast, the claimed invention requires the targeting domain to bind the same substrate which the catalytic domain cleaves. Therefore, combining Whitcomb with Holvoet, a skilled artisan will at best generate a fusion protein with a mesotrypsin protease domain and a fibrin-specific antibody targeting domain, which does not fall within the scope of any of the pending claims.

Chen relates to a so-called "targeted enzyme" that "comprises a substrate recognition site and has been modified from a pre-targeted enzyme to comprise one or more targeting sites, each targeting site comprising one or more variant sequences, and to bind to a target with higher affinity than the corresponding pre-targeted enzyme binds the target under like conditions. ... Targeted enzymes of the invention do not include enzymes with a targeting site that consists of a polypeptide or other target-binding molecule that is attached to the N- or C-terminus of the pre-targeted enzyme (e.g., as in a histidine tagged protein or a fusion protein), a targeted enzyme whose only target is a monoclonal antibody, or a targeted enzyme made by increasing or optimizing the binding of a pre-targeted enzyme to a substrate of a reaction catalyzed by the pre-targeted enzyme" (emphasis added).

In other words, the "targeted enzyme" of Chen merely modifies so-called "variant sequences" on the pre-targeted enzyme, such that after the modification, the enzyme acquires the ability to bind a target that the enzyme previously cannot bind. In doing so, Chen explicitly states that the "targeted enzyme" does not include any fusion protein created by fusing a targeting domain to (the catalytic domain of) the enzyme (see emphasis above), as is recited in the claimed invention.

Thus combining Chen with Whitcomb would result in modifying the "variant sequences" on mesotrypsin to create a targeted mesotrypsin that binds substrates mesotrypsin previously cannot bind. However, the resulting "targeted mesotrypsin" is not a fusion protein with discrete and heterologous protease domain and targeting domain, as recited in the claimed invention. Thus the combined teaching of Chen and Whitcomb still fails to teach or suggest all the limitations of the claimed invention.

Likewise, Davis teaches away from the claimed fusion protein by suggesting that the targeting moiety should be coupled to the catalytic domain through chemical cross-linking to a Cysteine sulfur in polypeptide comprising the catalytic domain. *See* page 6, 1st full paragraph, and the paragraph bridging pages 21 and 22. Thus combining Davis with Whitcomb would result in a mesotrypsin catalytic domain chemically cross-linked to a targeting domain via a natural or engineered Cys residue on the catalytic domain. In contrast, the claimed invention relates to a fusion protein (*see* Claim 1) or an immunoglobulin fusion complex (*see* Claim 41).

Therefore, the combined teaching of Davis and Whitcomb still fails to teach or suggest all the limitations of the claimed invention.

In summary, other than mere conclusory statements, the Office Action has failed to identify any reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. In addition, even assuming for the sake of argument that Whitcomb or Sallberg can be combined with Holvoet, Davis, or Chen, the combined teaching still fails to teach or suggest all the limitations of the claims in each case. Therefore, a *prima facie* case of obviousness has not been established. Reconsideration and withdrawal of the rejections under 35 U.S.C. § 103(a) are respectfully requested.

Double Patenting Rejection

The Office Action states that Claims 1, 4-25, and 30-41 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claim 1, 4-25, and 30-41 of the co-pending U.S. Application No. 10/792,498.

The Office Action also states that Claims 1, 4-25, and 28-41 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claim 1, 4-38, 40-46, 52-60, 66-104, 107-134 of the co-pending U.S. Application No. 10/650,592.

Applicants submit that, pursuant to MPEP 804, "[i]f the 'provisional' double patenting rejection in one application is the only rejection remaining in that application, the examiner should then withdraw that rejection and permit the application to issue as a patent [without filing a terminal disclaimer], thereby converting the 'provisional' double patenting rejection in the other application(s) into a double patenting rejection at the time the one application issues as a patent."

Applicants note that no claim has been issued in the co-pending application Nos. 10/792,498 and 10/650,592. Thus if the only rejection in the instant application is the provisional double patenting rejection, the Examiner should withdraw that rejection and permit the application to issue as a patent without requiring a terminal disclaimer.

If conflicting claims are first allowed in the co-pending U.S. Application No. 10/792,498 or U.S. Application No. 10/650,592, and appear in an issued U.S. patent, Applicants note that, pursuant to 37 C.F.R. § 1.130(b), a timely filed terminal disclaimer in compliance with 37 C.F.R. § 1.321(c) may be used to overcome the double patenting rejection. Applicants will submit a terminal disclaimer, if necessary, upon indication of allowable subject matter.

CONCLUSION

The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. The Director is hereby authorized to charge any other deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. **18-1945**, from which the undersigned is authorized to draw under Order No. **COTH-P02-001**.

Dated: May 22, 2007

Respectfully submitted,

By_____

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Soluble cytokine receptors: their role in immunoregulation

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ABSTRACT **A number of cytokine receptors exist in soluble form in the biological fluids of both animals and humans, a phenomenon that might have immunoregulatory implications in vivo. Although these soluble receptors specifically inhibit binding and activity of their respective cytokines in vitro, their actual function in vivo as cytokine inhibitors or as carrier proteins is unclear. Abnormalities in the production of these substances might contribute to the pathophysiology of immune and neoplastic diseases. Besides their role in regulating cytokine activity in vivo, soluble cytokine receptors hold significant potential for therapeutic use as very specific anticytokine agents and as indicators in diagnosis and assessment of immune parameters, prognosis, disease progression, response to treatment, etc., in a variety of autoimmune and malignant diseases. — Fernandez-Botran, R. Soluble cytokine receptors: their role in immunoregulation. *FASEB J.* 5: 2567-2574; 1991.**

Key Words: cytokine activity · cytokine inhibitor · carrier proteins · diagnosis · immunotherapy

CYTOKINES ARE A GROUP OF hormone-like regulatory molecules secreted by T lymphocytes, macrophages, and other cells. Their many regulatory roles include the control of cellular and humoral immune responses, inflammation, fever, chemotaxis, acute-phase responses, tumor regression, hemopoiesis, and many other functions (1-3). Although different cytokines have distinct activities, their effects are not restricted to particular cell lineages as was once thought, i.e., most can act on cells from different lineages at several stages along their development/differentiation pathway. A brief summary of the biological activities of selected cytokines is presented in **Table 1** (1, 4).

In vivo, cytokines do not act alone but in combination with other cytokines and stimuli. Different cytokines can have redundant effects on certain cells, and combinations of cytokines can be synergistic or antagonistic in their effects depending on the target cells (1-3). Because of such complexity and because much of the knowledge of the biologic activities of cytokines has been derived from in vitro studies, actual roles played by cytokines and regulation of their activity in vivo are not completely clear. Moreover, understanding mechanisms that regulate cytokine activity in vivo requires consideration of other parameters such as different microenvironments and anatomical compartmentalization.

Because of the potent and profound biological effects of cytokines, it is not surprising that their activities are tightly regulated, most notably at the levels of secretion and receptor expression. Additional regulatory mechanisms are provided by the concomitant action of different cytokines and the presence in biological fluids of specific inhibitory proteins and nonspecific carrier substances. The purpose of this work is to review our current knowledge of these inhibitory substances, especially soluble cytokine receptors, and discuss their potential role in regulation of cytokine activity in vivo, their significance in disease, and their potential use as immunotherapeutic agents.

REGULATION OF CYTOKINE ACTIVITY

Many different mechanisms play a role in the regulation of cytokine activity. This is accomplished at many levels: *1*) Regulation of cytokine secretion; *2*) Regulation of cytokine receptor expression; *3*) Regulation of the activity of one cytokine by other cytokines; and *4*) Regulation by soluble cytokine-binding factors and/or inhibitors.

Regulation of cytokine secretion

Most cytokines are not secreted constitutively but in response to antigenic, mitogenic, or other types of stimuli (1, 5). The induction of cytokine secretion by T cells is usually antigen-specific (6, 7), whereas secretion of macrophage-derived cytokines (interleukin 1, [IL 1],[1] IL 6, tumor necrosis factor-α [TNFα]) is dependent on stimuli such as endotoxin and other bacterial products, viruses, and other cytokines (8). The genes encoding cytokines and their receptors are under the control of multiple transcription factors and regulatory elements (5), which are usually not restricted to cytokine genes. Exogenous (soluble antigens, viruses) or endogenous (cytokines, cofactors) stimuli that elicit cytokine secretion can regulate the activity of transcription factors either by inducing their de novo expression such as in the case of the interferon-responsive factor, IRF 1 (9), which may regulate expression of a number of cytokine and noncytokine genes (5, 10), or by the posttranslational activation of factors such as NF-κB, which regulates expression of cytokine (IL 2, interferon γ, or IFN γ, IL 6), cytokine receptor genes (IL 2R), the κ-light chain immunoglobulin gene, and human immunodeficiency virus (HIV 1) (11). Not only is the synthesis of cytokines regulated by transcription factors, but cytokines themselves can also induce specific nuclear factors that influence transcription of other cytokine and noncytokine genes (5). IL 1, for example, induces the activity of NF-κB, which regulates expression of the genes encoding for IL 1, IL 2R, and IL 6.

Regulation of cytokine receptor expression

In contrast to the inducible nature of cytokine secretion, the expression of some cytokine receptors (i.e., IL 1R, IL 4R, IL 6R) appears to be constitutive, assuring the presence of

[1]Abbreviations: FGF, fibroblast growth factor; IL, interleukin; IL1ra, interleukin-1 antagonist; IFN-γ; interferon-γ; PDGF, platelet-derived growth factor; R, receptor; s, soluble; TNF, tumor necrosis factor; EGF, epidermal growth factor; NK, natural killer; HIV 1, human immunodeficiency virus; CSF, colony-stimulating factor.

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TABLE 1. *Biologic activities of some cytokines*

Cytokine	Secreting cells	Activities
Interleukin 1 (IL 1α, IL 1β)	Monocytic cells, T and B cell lines, NK cells, fibroblasts, endothelial cells, others	Proliferation of T and B cells; activation of macrophages; inflammation, fever
Interleukin 2	Activated T cells	T cell growth factor; cytotoxic T cell generation; B cell proliferation/differentiation; growth of NK and LAK cells
Interleukin 4	Activated T cells, mast cells, non-B-, non-T spleen cells	B cell activation/differentiation; T and mast cell growth; activation of macrophages
Interleukin 6	Activated T cells, macrophages, fibroblasts	B cell proliferation/differentiation; hybridoma/plasmacytoma growth factor; T cell activation; fever, inflammation
Interleukin 7	Bone marrow stromal cells	Lymphoid precursors differentiation; T cell proliferation
Interferon γ	Activated T cells	Antiviral activity, B cell proliferation/differentiation; antagonist of IL 4 (on B cells); activation of macrophages
Tumor necrosis factor α (TNFα)	T cells, macrophages, NK cells	Tumoricidal activity, B cell growth and differentiation; procoagulant activity; inhibition of lipoprotein lipase

responding cells (8, 12–15). Activating stimuli nonetheless usually lead to up-regulation of the number of such receptors, with subsequent amplification of the response (13). Expression of other receptors, such as the p55 subunit of the IL 2R, is dependent on cell activation (3, 6) and is transcriptionally regulated in much the same manner as cytokine genes (5). IL 2Rs (high affinity) are formed by interaction of at least two subunits, p75 and p55, which can independently bind IL 2 with intermediate and low affinities, respectively (16, 17). Thus, whereas resting T cells express p75 (the intermediate affinity IL 2R) and are able to respond to IL 2, albeit at very high doses, antigenic stimulation results in the induction of p55 expression and formation of the high-affinity IL 2R, enabling activated T cells to respond to very low concentrations of IL 2 (3). The fact that some cytokines can affect the activity of other cytokines results, in some cases, from their ability to regulate expression of the receptors for those other cytokines (18).

Regulation by other cytokines

Some cytokines have the ability to induce secretion of other cytokines or to up-regulate expression of their own or other cytokine receptors. In certain systems, the effects of some cytokines are antagonized by the effects of other cytokines (1). In the mouse, for example, INF γ is a well-known antagonist of the activities of IL 4 on B cells (1, 19) (2), inhibiting IL 4-induced class II and CD23 expression, proliferation, and IgG_1 and IgE secretion. On the other hand, the IFN γ-induced secretion of IgG_{2a} is inhibited by IL 4 (1). These effects are not mediated through competition at the level of surface receptors, as binding of IL 4 to B cells is unaffected by IFN-γ (13–15). The effects of IL 4 on other cells such as T cells, mast cells, and macrophages appear to be unaffected by IFN-γ.

Soluble inhibitors

Many papers have documented the existence of factors able to inhibit the activity of certain cytokines in biological fluids or in tissue culture supernatants. Such factors have been given the name inhibitors and are usually cytokine-specific in their action. Mechanisms responsible for the inhibitory effects of these factors are varied, although they usually interfere with the formation of a cytokine/receptor complex, thereby inhibiting subsequent signal transduction. In this regard, these cytokine inhibitors can be divided, based on their mode of action, into *1*) receptor-binding antagonists which inhibit binding of a cytokine to its receptor by competing for the same binding site (20, 21); and *2*) cytokine-binding proteins, which inhibit the binding of a cytokine to its receptor by binding to the free cytokine (22–26). Cytokine inhibitors that do not appear to interfere with the binding of cytokines to their receptors have also been described (27–29). The bases for activities of such inhibitors are less well characterized and will not be discussed further in this review. In addition to the specific cytokine-binding factors, serum proteins such as α2-macroglobulin (30) have been documented to bind a number of cytokines, including IL 1 (31) and IL 6 (32), and may act as inhibitors or as carriers for these cytokines in circulation.

Receptor-binding antagonists

Receptor antagonists bind specifically to a cytokine receptor but are themselves devoid of biologic activity. Thus, these types of molecules compete with the biologically active cytokine for binding to the same membrane receptor. To date, the only example of this kind of inhibitor is the human IL 1 receptor antagonist (IL 1ra), recently described by Hannum et al. (20) and cloned by Eisenberg et al. (21). The IL 1ra is produced by monocytic cells (the same cells that se-

crete IL 1) grown on adherent immune complexes or IgG. The basis for the inhibitory effect of the IL 1ra lies in its ability to bind to IL 1 receptors (IL 1Rs), inhibiting the binding and subsequent signaling of both forms of IL 1 (IL 1α and IL 1β). Although clearly different from IL 1, sequence analysis indicates that the IL 1ra possesses some structural similarities with IL 1β (26% homology). Comparison of the amino acid sequences of both IL 1 forms and IL 1ra might help identify those regions required for receptor binding and those for signaling. Based on similar physicochemical properties, the molecule cloned as the IL 1ra might be identical with other IL 1 inhibitors previously reported to be present in the urine of febrile patients or patients with myelomonocytic leukemias (20, 21). The IL 1ra is likely to act as a negative regulator of IL 1 activity in vivo.

Cytokine-binding inhibitors

Many cytokine inhibitors exert their action by binding to the free cytokine in solution, precluding its binding to specific membrane receptors. Availability of specific antireceptor antibodies, and cloning and expression of the genes coding for cell-surface cytokine receptors, have allowed identification of some of these cytokine-binding proteins as soluble forms of the membrane-bound receptors. These soluble receptors represent truncated forms of the membrane receptors that lack transmembrane and intracytoplasmic domains, but retain the ligand-binding extracellular portion (24, 25, 33). The affinities of soluble receptors for their ligands are usually comparable to those of the membrane receptors (24, 25). These soluble receptors may arise from proteolytic cleavage of the original membrane-bound receptor or by synthesis from separate alternatively spliced mRNAs coding for soluble vs. membrane forms (24, 25, 34, 35).

Because soluble receptors can compete with membrane receptors for binding of free cytokine, they are generally regarded as inhibitors. Indeed, interference with cytokine activity has been achieved with a number of natural or recombinant soluble receptors in vitro (24, 25, 35, 36). Soluble receptor/cytokine complexes have also been reported to be able to trigger a biological response in the case of IL 6R (8). Also, it is possible that binding of cytokines by soluble receptors in vivo might protect them from proteolytic inactivation, thereby increasing their half-life in the circulation (i.e., carrier proteins).

Soluble receptors for several cytokines have been detected in biologic fluids of humans and mice, including the low-affinity subunit (p55) of IL 2R (33, 37–41), the IL 6R (42), IFN-γR (42), and TNF-R (26, 43, 44). In addition, cDNAs encoding soluble forms of the murine IL 4R (24) and human IL 7R (25) have been described. Our laboratory has also reported the presence of an IL 4-binding protein (IL 4BP), with properties similar to the recombinant sIL 4R, in the biologic fluids of mice (23).

Soluble IL 2Rs

Of the two subunits that compose the high-affinity IL 2R, the larger (p75) subunit is constitutively present on resting T lymphocytes, natural killer (NK), and other types of cells, whereas expression of the smaller (p55) subunit is dependent on cell activation (3) and leads to formation of the high-affinity IL 2R (K_d: 10^{-11} M) (3), enabling activated cells to be triggered by low concentrations of IL 2. In addition to normal activated cells, a number of neoplastic or HTLV-I-infected cells have been shown to constitutively express the p55 chain (45).

In addition to membrane expression of the p55 chain of the IL 2R, activated or HTLV-I-infected T cells secrete a soluble form of this subunit (sIL 2R) (38–40, 46). This truncated form of p55 is smaller (40–50 kDa), but retains the ability to bind IL 2 with roughly the same affinity as the membrane form (34). Although the relatively low affinity of the sIL 2R (when compared with the biologically active high-affinity IL 2R [p75-p55]) has been difficult to reconcile with biologic activity as an IL 2 inhibitor (40, 41), when recombinant or purified sIL 2R has been used in high enough concentrations it can specifically inhibit IL 2 activity in vitro (33, 47).

In vivo, small concentrations of sIL 2R are normally present in the serum and urine of both animals and humans (33, 37–41). Increased concentrations of sIL 2R (sometimes up to 10- to 100-fold over normal levels), however, have been observed in a wide variety of autoimmune, viral, parasitic, and neoplastic diseases (45, 48–52). As production of sIL 2Rs has been correlated with cellular activation, the high levels found in the serum of patients suffering from such diseases probably result from generalized T cell activation. Whether at these elevated concentrations sIL 2R might act as an inhibitor of IL 2 activity in vivo, perhaps contributing to the immune suppression observed in some of these diseases, remains a matter of speculation. Nonetheless, evaluation of the serum levels of sIL 2R has proved valuable as an indicator of the severity, prognosis, or efficacy of treatment in a number of autoimmune and neoplastic diseases. In addition, serum levels of sIL 2R appear to correlate directly with the level of progression in HIV-induced disease (52).

Honda et al. (53) have recently described the presence of soluble forms of the intermediate-affinity IL 2R (p70) in culture supernatants derived from several human lymphoid cell lines and from mitogen-stimulated peripheral blood mononuclear cells, suggesting that both subunits of the IL 2R might exist in soluble form and that production of soluble p70 forms might also be related to cell activation. In addition, production of soluble p70 was found to be increased in HTLV-I-infected cell lines. These soluble forms retain their ability to bind IL 2, but are approximately 20- to 25-kDa smaller than the membrane receptors, reflecting the absence of transmembrane and intracytoplasmic domains.

Soluble IL 4Rs

Mosley et al. (24) reported the cloning of the murine IL 4R. The authors found three different types of cDNAs; one encoding a 140-kDa membrane-bound form of the IL 4R containing extracellular, transmembrane, and intracytoplasmic domains; a second type lacking most of the intracytoplasmic domain; and a third type that codes only for the extracytoplasmic domain. The latter type of cDNA contains a 114-bp insertion that introduces a termination codon before the transmembrane domain. This insertion may represent an alternate exon sometimes retained during splicing of the primary transcript to generate an mRNA coding for a soluble form of the IL 4R. COS-7 cells transfected with this type of clone secreted a soluble form of the IL 4R (sIL 4R) that had a similar binding affinity to that of the membrane-bound IL 4R form. In addition, the sIL 4R was able to inhibit the binding of ^{125}I-labeled IL 4 to CTLL cells and the IL 4-mediated proliferative response of these cells.

Consistent with the idea that sIL 4R can act as a competitive inhibitor of IL 4, recombinant sIL 4R has also been reported to inhibit all the effects of IL 4 on B lymphocytes, including the increased expression of MHC class II

TABLE 2. *Summary of the properties of soluble cytokine receptors and inhibitors*

Soluble receptor/antagonist	Properties	Inhibition of activity
IL 1 antagonist (IL 1ra)	Binds to IL 1Rs, prevents binding of IL 1 produced by macrophages	Yes
IL 1-binding protein	Binds to IL 1β with higher affinity than to IL 1 α (soluble IL 1R?)	Not known
sIL 2R (p55)	Binds IL 2 with low affinity (K_d: 10^{-8} M); produced by activated T cells	Yes (if in high concentration)
sIL 2R (p70)	Binds IL 2, produced by lymphoid cell lines, activated T cells, and HTLV-I-infected cells	Not known
sIL 4R	Binds IL 4 with high affinity (K_d : 5×10^{-11} M); inhibits binding	Yes
sIL 6R	Binds IL 6	Not known
sIL 7R	Binds IL 7; inhibits binding	Not known
sIFN γR	Binds IFN-γ	Not known
sTNF R	Binds TNFα with higher affinity than TNFβ	Yes

molecules and CD23, proliferation in the presence of anti-Ig antibodies, and secretion of IgG_1 and IgE by LPS-stimulated B cells (36). Although cDNAs encoding the soluble form of IL 4R were isolated from more than one cell line and were detected by Northern blotting in different tissues, it is not yet clear whether significant amounts of sIL 4R are produced in vivo from this type of RNA.

Our laboratory recently reported the presence in murine biological fluids (serum, urine, ascitic fluid) of a protein with the ability to bind IL 4 and prevent its binding to IL 4R$^+$ cells (23). This protein binds IL 4 with an affinity comparable to that of the IL 4R, is specific for IL 4, and shows species specificity in that it does not bind human IL 4. The IL 4BP can also specifically inhibit the activity of IL 4 on an IL 4-dependent T cell line or on normal B cells. Besides the similar affinity for IL 4, the M_r of IL 4BP is similar to that reported for the recombinant sIL 4R produced in COS-7 cells, suggesting the possibility that the IL 4BP is the natural, soluble form of the IL 4R. Based on equilibrium binding experiments, it has been determined that the concentration of IL 4BP in serum is approximately 20 ng/ml, equivalent to a concentration of 0.5×10^{-9} M. Recent results from our laboratory indicate that recombinant sIL 4Rs and purified IL 4BP generate similar one-dimensional (Cleveland) peptide maps upon digestion with several proteases, and that a rat anti-murine IL 4R monoclonal antibody is able to completely inhibit binding of ^{125}I-labeled IL 4 to purified IL 4BP (R. Fernandez-Botran and E. S. Vitetta, in press), demonstrating that IL 4BP is a soluble form of IL 4R.

In contrast to the low affinity of the sIL 2R for its ligand, the high affinity of IL 4BP (K_d: 6×10^{-11} M) and its relatively high concentration in serum suggest that this protein plays a significant role in the regulation of IL 4 activity in vivo by competing with cellular IL 4Rs for binding of IL 4, perhaps helping to keep the action of IL 4 relatively confined to the site of secretion. Nonetheless, IL 4BP might also function as a transport protein for IL 4 in circulation. Studies from our laboratory have suggested that in contrast with the relatively slow dissociation kinetics of IL 4 from membrane IL 4Rs, the dissociation of IL 4 from the soluble IL 4BP at 37°C is fast, allowing the bound IL 4 to rapidly dissociate from the IL 4BP and bind to IL 4R$^+$ cells (R. Fernandez-Botran and E. S. Vitetta, in press). This property may indicate that the IL 4BP and perhaps other soluble cytokine receptors might function both as inhibitors and as carriers for their cytokines in circulation. The eventual effect of the soluble receptor would then depend on the relative concentrations of cytokine, the soluble receptor, and receptor-bearing cells. Thus, the relatively higher concentration of the soluble receptor in circulation might result in an inhibitory effect on cytokine activity, whereas in a lymphoid organ the relatively higher concentration of membrane receptors might shift equilibrium toward the cytokine being bound and acting on the cells.

Soluble IL 7Rs

Interleukin 7 is a cytokine involved in regulation of lymphopoiesis. Interleukin 7 also has growth factor activity on cells of T and B lineages at different stages of maturation (25). The IL 7R has been cloned recently and shown to be a member of the hemopoietin receptor family (25). Similar to IL 4R, several clones coding a soluble IL 7R were isolated in addition to cDNA clones encoding the membrane-bound receptor. In the case of the clones coding the soluble IL 7R, a deletion results in an altered translational reading frame, causing addition of 27 amino acids before termination. Among the sequences deleted are those encoding for the transmembrane portion. As predicted, COS-7 cells transfected with such clones produce a secreted form of the IL 7R that is able to bind IL 7 in solution and to inhibit binding of ^{125}I-labeled IL 7 to IL 7R$^+$ cells. Taken together, studies of the IL 4R and IL 7Rs suggest that a number of mechanisms might be operative in giving rise to messages encoding the soluble forms of cytokine receptors.

Soluble TNF-Rs

Tumor necrosis factor α (TNFα) and lymphotoxin (TNFβ) are two related cytokines secreted primarily by activated macrophages or lymphocytes, respectively. Both forms bind to the same receptors and share many biologic activities, including effects in inflammation, anti-tumor and anti-viral activities, endotoxic shock, cachexia, angiogenesis, and

mitogenesis (35). Two distinct TNF-Rs have been identified: a myeloid cell-type receptor (K_d: 7×10^{-11} M) and an epithelial cell-type receptor (K_d: 3×10^{-10} M) (54). Soluble TNF-binding proteins have been identified in normal human urine (26, 43) and in the serum of human patients suffering from various forms of cancer but not in the serum of normal controls (44). These TNF-binding proteins represent soluble forms of both types of membrane TNF-Rs by sequence homology and reactivity with specific antibodies (35) and are able to inhibit binding and activity of TNF in vitro (26, 43, 44). It is possible that the presence of soluble TNF-Rs in the serum of patients with malignancies is an important mechanism whereby tumor cells protect themselves from the toxic effects of TNF. It remains to be determined whether any correlation exists between the levels of soluble TNF-Rs in serum and tumor progression and/or metastasis. Whereas the membrane TNF-Rs bind TNFα and TNFβ with similar affinity, the soluble TNF-Rs have a higher affinity for TNFα (26, 35).

Other soluble receptors

The presence of proteins in normal human urine with the ability to bind either IL 6 or IFN-γ has been reported recently after purification on immobilized IL 6 or IFN-γ, respectively (42). The identity of these proteins with the respective IL 6R and IFN-γRs was accomplished by comparison of the amino-terminal sequences of the IL 6BP with that reported for the membrane IL 6R and by reactivity of the IFN-γ BP with anti-IFN-γR antibodies.

A soluble protein produced by activated mononuclear cells and with the ability to specifically bind IL 1β, but not IL 1α, has been described by Symons et al. (22). Whether such protein is related to the 80-kDa IL 1R or to the newly described B cell 60-kDa IL 1R has not yet been determined.

Soluble receptor forms are not exclusive of cytokine receptors. Precedent exists for natural forms of soluble receptors for several hormones, including colony-stimulating factor (CSF) (55), epidermal growth factor (EGF) (56), insulin-like growth factors (57), and growth hormone (58). Moreover, soluble forms of other membrane molecules, such as MHC class I (59) and CD8 (60), have also been described. Widespread distribution of these molecules suggests that the presence of soluble receptors in biological fluids might be part of a general regulatory mechanism for the activity of soluble mediators in vivo. A summary of the properties of soluble cytokine receptor and antagonists is presented in **Table 2.**

Generation of soluble cytokine receptors

In the case of some cytokine receptors, such as sIL 2R (p55) and sTNF-R, the secreted forms are thought to arise by proteolytic cleavage of membrane receptors (shedding) (34, 35). In other cases, however, separate cDNAs encoding membrane vs. soluble receptor forms have been isolated (i.e., IL 4R, IL 7R), (24, 25), which suggests that some secreted receptors may arise by translation from alternatively spliced messages. It is not yet clear whether the naturally occurring form of soluble IL 4Rs (IL 4BP) arises from an alternatively spliced message or through proteolysis of membrane receptors. Little is known about the factors that regulate differential expression of membrane vs. soluble receptor forms.

Nonreceptor inhibitors

In addition to soluble receptors, serum or urinary proteins not characterized as related to cellular receptors have been reported to be able to bind certain cytokines and in some cases to inhibit their activity, such as Uromodulin, a protein present in the urine of pregnant women and shown to bind IL 1 (27). Other proteins have been implicated as transport proteins rather than as inhibitors for some cytokines. An example is serum α2-macroglobulin, a major protein in serum, which in addition to acting as a protease inhibitor and carrier has been reported to bind a number of cytokines and hormones, including IL 1, IL 6, IL 2, PDGF, and FGFβ (30). Although it apparently does not affect the binding and activity of IL 1 (31), it has been reported to protect IL 6 from the action of proteases and has been suggested to act as an IL 6-transport protein in circulation (32). In contrast, α2-macroglobulin interferes with or leads to inactivation of other cytokines such as IL 2 and FGFβ (30). Although the exact role played by α2-macroglobulin in immunoregulation is far from clear, these results suggest that it might promote the activity of some cytokines, such as IL 1 and IL 6, while acting as an inhibitor for others. **Figure 1** illustrates some of the mechanisms involved in the regulation of cytokine activity in vivo.

SOLUBLE CYTOKINE RECEPTORS IN DISEASE

As mentioned previously, soluble forms of IL 2Rs, IL 4BP/IL 4Rs, IL 6Rs, IFNγ-Rs, and TNF-Rs exist in biolog-

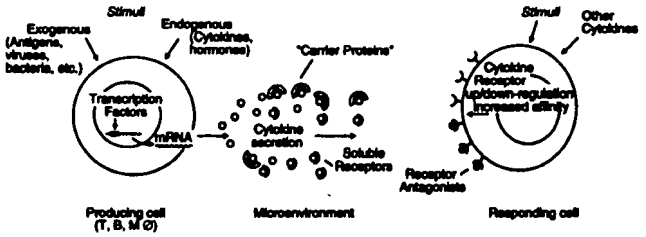

Figure 1. Mechanisms involved in regulation of cytokine activity in vivo. Cytokine synthesis and secretion are dependent on stimulation of the cytokine-producing cells. Stimuli may be exogenous (antigens, microorganisms, endotoxin, etc.) or endogenous in nature (cytokines, hormones). Stimulation by these agents results in the activation of transcription factors (either by de novo synthesis or post-translational mechanisms), thus inducing transcription of cytokine genes and subsequent translation and secretion. Once released into the microenvironment, cytokines can interact directly with specific receptors present on the membrane of responding cells, with carrier proteins such as α 2-macroglobulin or with specific soluble receptors or binding proteins. The role of carrier proteins may be to transport cytokines in the circulation and to inhibit their degradation and/or excretion through the kidneys, prolonging the half-life of the active cytokines. Soluble receptors inhibit binding of cytokines to their membrane receptors and can thus act as competitive inhibitors, maintaining the action of cytokines relatively restricted to their site of secretion. A cytokine-carrier role for soluble receptors, however, is also possible. In any case, the amount of free cytokine available for binding to cell membrane receptors is determined by the relative concentrations of cytokines, soluble receptors, and membrane receptors. The binding of cytokines to their receptors on target cells may also be competitively inhibited by receptor antagonists that bind to the same receptors but are devoid of biologic activity (i.e., IL 1ra). Several factors can also affect responsiveness of the target cells to a cytokine, such as the state of activation and number and affinity of specific receptors. Stimulation by a variety of agents might result in the up/down-regulation of the number of specific cytokine receptors or in the synthesis of additional receptor subunits with the effect of increased receptor affinity and heightened responsiveness.

ical fluids in humans or animals. It is likely that conditions that affect the immune system and lead to activation, proliferation, or death of cells that secrete these receptors would alter the normal levels of these proteins. Alternatively, alterations in the relative levels of soluble cytokine receptors may not only reflect such alterations, but may themselves play a role in the pathophysiology of immune diseases. For example, increased levels of soluble TNF-Rs in the circulation might lead to inactivation of TNF, resulting in enhanced tumor cell growth and even metastasis. Increased levels of soluble IL 4Rs or even IL 2Rs might, on the other hand, interfere with IL 4 or IL 2 activity, respectively, leading to the decreased immune function usually observed in patients suffering from a number of autoimmune or malignant diseases. Understanding the relationship between disease and soluble cytokine receptors will require a basic knowledge of their immunoregulatory effects and a careful study of their levels in normal and pathologic conditions.

To date, the only extensive information in this regard comes from studies of sIL 2R. Levels of sIL 2R in serum appear to correlate with the level of activation of T cells or the load of IL $2R^+$-expressing malignant cells in vivo. Thus many reports have related the levels of sIL 2R and the severity or prognosis of autoimmune and malignant diseases. **Table 3** summarizes this information.

THERAPEUTIC POTENTIAL

Since the cloning of genes encoding the different cytokines and the development of anti-cytokine and anti-cytokine receptor monoclonal antibodies, much interest has been directed toward immune therapy with recombinant cytokines or cytokine-stimulated cells, especially with regard to treatment of malignant and immune deficiency states. In other pathological conditions, however, it is desirable to suppress the immune system (i.e., avoidance of graft rejection, autoimmune diseases, hypersensitivity). Currently this is accomplished through nonspecific immunosuppressive drugs such as antimetabolites or steroids, or with relatively more specific drugs such as Cyclosporin A. However, these may inhibit secretion of a number of cytokines as well as having other nonspecific effects.

Ideally, recombinant soluble receptors could provide the means for specific and potent immunosuppression of the effects of a particular cytokine without affecting the actions of others. Soluble receptors would be superior to anticytokine antibodies in that they have at least 100- to 1000-fold higher affinities and are smaller, allowing better distribution; they do not have structures reacting with other cells, such as the Fc portion of immunoglobulins, and would not be recognized as foreign by the immune system, such as in the case of monoclonal antibodies raised in different species, avoiding elicitation of an immune response.

As examples, therapy with recombinant soluble receptors might be of value in treating malignant diseases in which tumor cells use cytokines as autocrine growth factors, such as a number of lymphoid malignancies (IL 2) and multiple myelomas (IL 6). Therapy with recombinant sIL 4Rs might be useful in treating hypersensitivity, owing to the dependence of IgE secretion on IL 4; and therapy with recombinant IL 1Rs might be useful in the treatment of inflammatory diseases such as rheumatoid arthritis. In vivo administration of sIL-1R has already been shown to have profound inhibitory effects on the development of in vivo alloreactivity (61). FJ

TABLE 3. *Conditions in which elevated serum levels of sIL 2Rs have been reported*

Conditions	Examples
Malignancies	Hematologic, lymphoid, solid tumors and metastases
Viral diseases	HTLV-I, HIV, hepatitis, measles
Parasitic diseases	Malaria
Bacterial diseases	Tuberculosis
Autoimmune diseases	Rheumatoid arthritis, systemic lupus erythematosus, atopic eczema
Transplant rejection	
Others	Type I diabetes, thermal injury, after prolonged exercise

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